PHOSPHOPROTEOMIC PROFILING ANALYSIS IN PEDIATRIC ACUTE LEUKEMIAS AND IN SOLID TUMORS OF THE ADULT

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Phosphoproteomic profiling of pediatric B-ALL identifies in MLL patients a new signalling pathway for targeted therapy

We analyzed 120 pediatric patients affected with B-cell AL (B-Acute Lymphoblastic Leukemia) by Reverse Phase Protein Arrays (RPPA). Leukemia cells from bone marrow aspirates were stored in liquid nitrogen in the Bio Bank of the Laboratory of Pediatric Oncohematology in Padova. Clinical data, such as immunophenotype, outcome, response to therapy and chromosomal translocations, were collected for all the patients.

Patients without known genomic aberrancies were compared to MLL rearranged patients that usually have a very poor outcome in spite of high-dose chemotherapy. MLL rearranged patients show an activated pathway that, through the phosphorylation of AMPK, leads to the activation of eNOSIII and finally Bcl-2. Bcl-2 is a prosurvival and antiapoptotic protein that is able to grant a survival advantage to the blast cells. Thus, we tested the effects of an AMPK inhibitor (Calbiochem) on the survival of three human cell lines: RS4;11 and SEM (MLL B-cell ALs with t(4;11)) and REH (B-cell AL with t(12;21)). We observed that treated RS4;11 and SEM cells undergo more apoptosis than REH cells: 68% of SEM cells and only 35% of RS4;11 cells survived AMPK inhibition, while 95% of REH cells were insensitive to treatment. To check the inhibition of the AMPK pathway after Compound C treatment, we performed Western Blot experiments and densitometric analyses on treated and untreated RS4;11 and SEM cells: treatment with Compound C effectively inactivates AMPK β and eNOS III after eight hours. Importantly, at 24 hours the inhibition of AMPK leads to the decrease of activated Bcl-2 S70 levels, thus removing this apoptosis block. This means that the activation of AMPK contributes to the survival of MLL leukaemia cells, since inhibition of AMPK induces death in these cancer cells. Our data strongly encourages further studies of AMPK inhibitors as potential new drugs for the treatment of MLL leukaemia patients. The discovery of the molecular signal pathway portrait in subgroups of patients could play an important role for individualizing therapy and improving treatment outcome.

Reverse Phase Protein Assay (RPPA) defines specific proteomic patterns in childhood Acute Lymphoblastic Leukemia (ALL)

Although current chemotherapy protocols reach an event free survival (EFS) greater than 70%, the remaining cases relapse and ALL is still the first cause of death in children with cancer. Several efforts have been made to better understand the underlying mechanism causing ALL. Current chemotherapy protocols have reached a plateau of effectiveness. The detection of

minimal residual disease (MRD) during treatment course will definitively lead us to stratify children with ALL in standard (SR), intermediate (MR) and high (HR) risk, respectively. In the latter group we included patients with a prednisone poor response, with t(9;22) and t(4;11) (HR by default). Although this better definition of therapeutic response, the majority of relapses are included in the MR, suggesting that we are treating different forms of ALL. For this reason new biomarkers and molecular targets are needed.

We analyzed 81 diagnostic samples (peripheral blood and/or bone marrow) with B-precursor ALL, diagnosed and treated at our centers (Catania and Padova) and enrolled in AIEOP-LLA 2000 Protocol. Multiplex and phosphoproteomic analyses (RPPA) were used to interrogate the expression of 81 phosphorylated or native protein endpoints. We evaluated Pro- and Anti-Apoptotic, Protein Kinase, and Growth factor receptor cell signaling pathways. For statistical purposes, we considered the following characteristics: age at diagnosis, gender, immunophenotype, karyotype, white blood cell count, and percentage of blast at diagnosis, response to prednisone, level of MRD at day 33 (TP1) and day 78 (TP2) during Induction phase. Comparing several subgroups for gender, WBC count (higher *vs* less than 50.000/mL), immunophenotype (Common *vs* others), karyotype (t(12;21) positive *vs* negative), prednisone good (PGR) *vs* poor (PPR) responders, analysis of MRD (SR *vs* MR, MR *vs* HR, SR *vs* HR, MR *vs* MR relapsed) and diagnoses *vs* relapses, we did not find any statistically significant datum. Conversely, we found a statistically significant, higher expression of FosB and Annexin II in the group of children with WBC >50.000/mL (p value <0.05). Our study showed that in the apoptotic pathway, we detected a higher expression of all the pro-apoptotic proteins [Bax, SMAC/DIABLO, Caspase 7 total and cleaved (D198)], a low expression of anti-apoptotic proteins (Bcl-xL, XIAP) associated with a reduction or a lack of PARP total or cleaved (D214). We also found that a specific pattern of expression (higher PTEN (S380), PARP cleaved, Caspase 7 cleaved, PDK (S241), PKAc (T197), p90RSK (S380), MEK ½ (S217-221), IKBA (S32), GRB2; lower Beta Catenine) selected a subgroup of children (n. 9) with better survival when compared with other cases (p value <0.05). Our preliminary data showed that RPPA associated by a confirmatory Western blot analysis, is a reliable strategy for identification of biomarkers and/or molecules for targeted therapy. Application of this methodology on specific subgroup of cases with ALL will lead us to identify new therapeutic strategies.

Genomic analysis of childhood ALL cases with PTPN11 mutation

The PTPN11 gene encodes SHP-2, a non-receptor tyrosine phosphatase involved in many signalling pathways, which has a key role in development and hematopoiesis. Germline PTPN11 mutations are the main cause of Noonan Syndrome, a developmental disorder often associated with hematologic abnormalities, while somatic PTPN11 mutations were found mainly in paediatric leukaemia, and with a lower frequency in adult leukaemia and solid tumours. These mutations determine a gain-of-function, causing the hyperactivation of SHP-2 by destabilizing its inactive conformation, although distinct mutants have different functional alterations. In paediatric ALL (acute lymphoblastic leukaemia), our group found 7% mutations in the B-lineage subgroup, and no mutations in T-lineage ALL; moreover, we found PTPN11 and RAS mutations to be mutually exclusive, and none of the main chromosomal translocations associated with PTPN11 mutations.

The aim of this project was to search for a specific signature of differentially expressed genes in mutated patients, and to correlate it with the hyperactivation of signalling pathways. We analyzed 80 diagnostic samples with T-precursor ALL, diagnosed and treated at centers Monza and Padova. Multiplexed phosphoproteomic analysis (RPPA) was used to interrogate the

expression of 30 phosphorylated or native protein endpoints. We evaluated Pro- and Anti-Apoptotic, Protein Kinase, and Growth factor receptor cell signaling pathways.

Publications of the project

Accordi B, Espina V, Milani G, te Kronnie G, Liotta LA, Petricoin III EF, Garaci E, Basso G. Analisi del profilo fosfoproteomico delle B-ALL pediatriche con Reverse Phase Protein Arrays. *Haematologica* 2007;92(4).

Accordi B, Espina V, te Kronnie G, Liotta LA, Petricoin III EF, Basso G. Phosphoproteomic profiling of pediatric B-ALL patients using Reverse Phase Protein Arrays. *Haematologica* 2007;92(1).

Accordi B, Espina V, VanMeter AJ, Milani G, Sciro M, Giordan M, te Kronnie G, Petricoin E III, Liotta LA, De Maria R, Garaci E, Basso G. Pediatric B-ALL with MLL- rearrangement exhibits AMPK pathway activation and its targeted inhibition induces apoptosis. *Blood*. Sottomesso.

Espina V, Edmiston KH, Heiby M, Pierobon M, Sciro M, Merritt B, Banks S, Deng J, VanMeter AJ, Geho DH, Pastore L, Sennesh J, Petricoin III EF, Liotta LA. A Portrait of Tissue Phosphoprotein Stability in the Clinical Tissue Procurement Process. *Mol Cell Proteomics* 2008;7(10):1998-2018. Epub 2008 Jul 30.

Tumino M, Accordi B, Sciro M, Giordan M, te Kronnie G, Basso G, Lo Nigro L. Reverse phase protein assay (RPPA) defines specific proteomic patterns in childhood acute lymphoblastic leukemia (ALL). Manuscript in preparation.

Patents

Accordi B, Basso G, Liotta L, Petricoin E, te Kronnie G; inventors. AMPK Pathway as a Theranostic Indicator for Mixed Lineage Leukemia (MLL). Application number 61/064,692. March 20, 2008.

Operative Unit held by Dr. Donato Nitti

Studies about phosphoprotein cell signaling analysys of solid neoplasms performed in our Unit are:

- Breast cancer: we demonstrated the utility of the reverse phase protein array to analyze the phosphoproteomic profile in 25 patients with metastatic breast cancer before starting protein kinase inhibitor therapy and during treatment.
- Colorectal cancer:
  - The analysis of tumor biopsy of 15 patients with rectal cancer before starting neoadjuvant chemotherapy demonstrated a specific signaling pathway of the non responder patients.
  - The analysis of 34 matched colorectal cancer and synchronous liver metastases compared with 18 colorectal cancers without distant metastases revealed specific signal pathways activated in liver metastases that are clearly distinct from those activated in the primary colorectal cancers.
- Gastric cancer: we are performing an analysis of cell signaling pathways from three sets of subjects: 20 healthy subjects (10 HP - and 10 HP + vacA and/or cagA); 20 patients with gastric cancer with lymph node metastases (N+) and 20 patients with gastric cancer without lymph node metastases (N-).

Publications of the project

Galdi F. Pierobon M, Mammano E, Pucciarelli S, Agostini M, De Marchi F, Canzonieri V, De Paoli A, Liotta LA, Petricoin EF III, Belluco C, Nitti D. Comprehensive phosphoproteomic signal pathway analysis for the identification of prognostic biomarkers for neoadjuvant therapy for rectal cancer. In: *99th AACR Meeting. Abstract*; 12-16 April 2008; San Diego, USA. Manuscript in preparation.

Pierobon M, Calvert V, Belluco C, Garaci E, Deng J, Lise M, Nitti D, Mammano E, Liotta L, Petricoin EF III. Multiplexed cell signaling analysis of metastatic and non-metastatic colorectal cancer reveal COX2-EGFR signaling activation as a potential prognostic pathway biomarker. *Clinical Colorectal Cancer* (in press).

Wulfkuhle JD, Speer R, Pierobon M, Laird J, Espina V, Deng J, Mammano E, Yang SX, Swain SM, Nitti D, Esserman LJ, Belluco C, Liotta LA, Petricoin EF III. Multiplexed cell signaling analysis of human breast cancer applications for personalized therapy. *J Proteome Res* 2008;7(4):1508-17.